RESOLUTION OF 5'-MONONUCLEOTIDASE AND NON-SPECIFIC PHOSPHATASE ACTIVITIES FROM SKELETAL MUSCLES

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1. Introduction

The 5'-nucleotidases (5'-ribonucleoside phosphohydrolase, EC 3.1.3.5) are widely distributed in animal and plant tissues and in microorganisms [1–10].

The occurrence of the enzyme activity in heart muscle was originally reported by Reis [1] and recently confirmed by Burger and Lowenstein [11]; its presence in skeletal muscle, however, has never been clearly demonstrated. Thus in 1940 Reis [2] described an enzyme activity catalyzing the dephosphorylation of IM-5'-P in muscle extracts from various sources under conditions in which β-glycerophosphate was hydrolyzed at a much slower rate. On the other hand Purzycka [12] and Kendrick-Jones and Perry [13] failed to detect appreciable dephosphorylation of AM-5'-P in rat skeletal muscle, at least under conditions in which the mononucleotide was acted upon by adenylic deaminase and myokinase.

In view of the paramount importance of adenine nucleotides in the biochemical events of muscular contraction, we have reinvestigated the problem of the 5'-nucleotidase occurrence in muscle. The present study reports on enzymes that hydrolyze 5'-mononucleotides in skeletal muscles from different sources.

Muscle 5'-nucleotidases can be readily separated from non-specific phosphatases by Sephadex G-100 gel filtration. Different ratios between 5'-nucleotidase and non-specific phosphatase are observed in muscle extracts from various animals. Furthermore the 5'-nucleotidases differ in their specificity towards 5'-mononucleotides according to their origin.

2. Materials and methods

2.1. *Materials*

5'-mononucleotides, ATP, ADP, phosphorylated sugars and 3-phosphoglyceric acid were obtained either from Sigma Chemical Company or from Boehringer Sohn. *p*-Nitrophenylphosphate (PNPP) was purchased from British Drug House, α-naphtylphosphate from Merck and β-glycerophosphate from Nutritional Biochemical Corp. Tris (Merck) was used as a buffer in most experiments.

2.2. *Preparation of muscle extracts*

Animals were killed by decapitation and bled, muscles from backs and legs were removed immediately, placed in ice-cold 0.25 M sucrose and washed several times with the same sucrose solution; they were cut into small pieces and homogenized with 4 volumes of cold water in a Waring Blendor. When the temperature approached 4°, the homogenization was halted and started again after cooling in ice. The total homogenization time was 10 min. After filtration through cheese-cloth, the resulting homogenates were centrifuged at 48,000 × *g* for 45 min and the supernatant fluids were withdrawn with a drawn-out dropper. They were then brought to 40% saturation with ammonium sulfate and centrifuged at 10,000 × *g*. The precipitates were dissolved in a volume of tris-HCl 0.05 M pH 7.2 corresponding to 1/50 of the original supernatant volume. Enzyme activities catalyzing the dephosphorylation of 5'-mononucleotides and PNPP were always found negligible in the super-

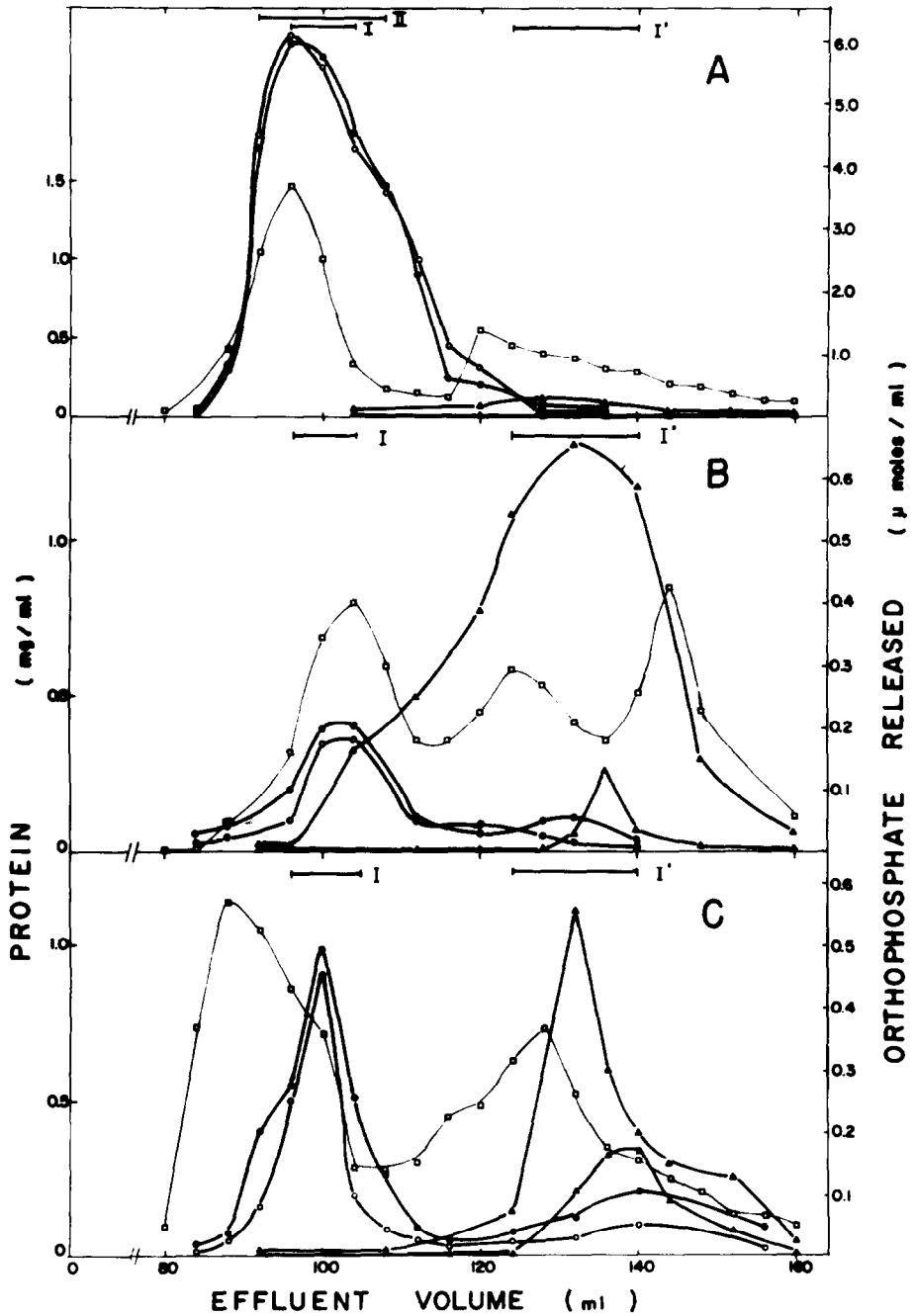

Fig. 1. Sephadex G-100 gel filtration of muscle extracts from guinea pig (A), rat (B) and chicken (C). For details of column separation, see section 2. Each fraction was analyzed for inosinate phosphatase and PNPPase in the presence and absence of Mg^{2+}. The assays contained 0.3 ml 0.2 M tris-HCl pH 8.5, 0.1 ml 10 mM substrate adjusted to pH 8.5, 0.3 ml of the G-100 column eluate and water to a final volume of 1 ml. The effect of Mg^{2+} was studied by including in the standard reaction mixture 0.030 ml of a 0.4 M solution of $MgCl_2$. Assays were started by addition of the eluate and incubations were conducted for 30 min. ○, orthophosphate released from IM-5′-P; △, orthophosphate released from PNPP; ●, orthophosphate released from IM-5′-P in the presence of Mg^{2+}; ▲, orthophosphate released from PNPP in the presence of Mg^{2+}; □, protein. All values have been corrected for blanks containing no substrate and for blanks containing no enzyme. The fractions indicated by I, I′ and II were used in the experiments described in tables 1 and 2, respectively.

natant fluids resulting from the ammonium sulfate precipitation.

2.3. *Sephadex gel filtration*

Two ml of the dissolved ammonium sulfate precipitate were applied to the top of a Sephadex G-100 gel column equilibrated with tris-HCl 0.05 M pH 7.2, and elution was carried out with the same buffer. The dimensions of the column were 2.4 × 50 cm. The flow rate was adjusted at 12 ml/h; fractions of 2.5 ml were collected.

Each fraction was assayed for protein and for enzyme activities catalyzing the dephosphorylation of IM-5′-P and PNPP, in the presence and absence of Mg^{2+}.

Results obtained with muscle extracts from guinea pig, rat and chicken are shown in fig. 1.

2.4. *Analytical methods*

The tubes were incubated at 37° for 30 min and the reaction was stopped by addition of half the volume of 35% perchloric acid. The precipitated protein was removed by centrifugation and orthophosphate was estimated in the supernatant fluid by the method of Fiske and SubbaRow [14].

Protein concentration in the enzyme preparations was measured by the spectrophotometric method, according to Warburg and Christian [15].

3. Results and discussion

The occurrence in muscle extracts of two distinct activities catalyzing the dephosphorylation of IM-5′-P and PNPP, respectively, can be demonstrated by separating the two enzyme activities by gel filtration on Sephadex G-100.

Dephosphorylation of IMP by the faster moving protein fraction is unaffected by addition of Mg^{2+}, while dephosphorylation of PNPP by the slower moving protein fraction is markedly stimulated by addition of the cation.

An examination of fig. 1 shows different levels of inosinate phosphatase and PNPPase in muscle extracts from various animals. This point is better illustrated in table 1, reporting the specific activities of the two enzymes, in the absence and in the presence of Mg^{2+}, as well as their relative ratios, found in muscle extracts of the animals tested.

Table 1

IMPase/PNPPase activity ratios of the column eluate of muscle extracts from guinea pig, rat and chicken.

Source		Specific activity		Ratio IMPase/ PNPPase
		IMPase	PNPPase	
Guinea pig	+ Mg^{2+}	0.570	0.014	40.7
	− Mg^{2+}	0.570	0.002	285
Rat	+ Mg^{2+}	0.008	0.042	0.19
	− Mg^{2+}	0.008	0.009	0.87
Chicken	+ Mg^{2+}	0.011	0.028	0.39
	− Mg^{2+}	0.010	0.007	1.43

Experimental conditions are described under fig. 1. Fractions I and I′ of fig. 1 were used as enzyme source. Specific activities are expressed as μmoles of orthophosphate released/min/mg protein.

Table 2

Relative rates of dephosphorylation of 5′-mononucleotides and other phosphorylated compounds catalyzed by the 5′-nucleotidase from muscle extract of guinea pig.

Substrate	Relative rate
Inosine-5′-monophosphate	100
Adenosine-5′-monophosphate	1363
Mixed isomers of 2′- and 3′-adenosine monophosphate	0.5
Cytidine-5′-monophosphate	134
Guanosine-5′-monophosphate	91
Uridine-5′-monophosphate	224
Xantosine-5′-monophosphate	25
p-Nitrophenylphosphate	0.4
α-Naphtylphosphate	0.1
β-Glycerophosphate	1.4
3-Phosphoglycerate	0.8
Fructose-1-phosphate	0.2
Fructose-1,6-diphosphate	2.3
Adenosine diphosphate	2.1
Adenosine triphosphate	4.3

Experimental conditions are described under fig. 1. Fraction II of fig. 1 was used as enzyme source.

It can be seen that the muscle extracts from guinea pig show the highest ratio of inosinate phosphatase to PNPPase activities, while the reverse is true for muscle extracts from rat which is practically devoid of inosinate phosphatase activity. The latter result could explain the findings of Purzycka [12] and Kendrick-Jones and Perry [13] who failed to detect

appreciable 5′-nucleotidase activity in rat muscle extracts.

The results discussed so far suggest that the dephosphorylation of IM-5′-P is catalyzed by a specific 5′-nucleotidase. This point was investigated further by incubating various 5′-mononucleotides and other phosphorylated compounds with the fraction eluted by gel filtration which displays inosinate monophosphatase activity. The results obtained with guinea pig muscle extracts are reported in table 2.

As shown in table 2, 5′-mononucleotides appear to be specific substrates for the guinea pig enzyme associated with the faster moving protein peak, UM-5′-P being the preferred substrate. 2′- and 3′-mononucleotides are practically not hydrolyzed. The classical substrates of non-specific phosphatase (PNPP, α-naphtylphosphate, β-glycerophosphate) and hydrolyzed at insignificant rate.

Among the phosphorylated sugars tested, only fructose-1,6-diphosphate is dephosphorylated at a significant rate. This might be attributed to a contaminating specific phosphatase. The same holds true for the slight, but significant dephosphorylation of ATP, even though purified preparations of 5′-nucleotidase are known to hydrolyze ATP at an appreciable rate [16].

Similar experiments were carried out with muscle extracts from other sources. AM-5′-P was found to be the preferred substrate, followed by UM-5′-P, GM-5′-P, IM-5′-P, CM-5′-P for chicken muscle extracts, and by IM-5′-P, GM-5′-P, UM-5′-P, CM-5′-P for rat muscle extracts.

Our results suggest that muscle 5′-mononucleotidases comprise an apparently large and diverse group of enzymes showing different specificities according to their origin.

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